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RESEARCH ARTICLE

# Assignment of a dubious gene cluster to melanin biosynthesis in the tomato fungal pathogen *Cladosporium fulvum*

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## Abstract

Pigments and phytotoxins are crucial for the survival and spread of plant pathogenic fungi. The genome of the tomato biotrophic fungal pathogen *Cladosporium fulvum* contains a predicted gene cluster (*CfPKS1*, *CfPRF1*, *CfRDT1* and *CfTSF1*) that is syntenic with the characterized elsinochrome toxin gene cluster in the citrus pathogen *Elsinoë fawcettii*. However, a previous phylogenetic analysis suggested that *CfPKs1* might instead be involved in pigment production. Here, we report the characterization of the *CfPKS1* gene cluster to resolve this ambiguity. Activation of the regulator *CfTSF1* specifically induced the expression of *CfPKS1* and *CfRDT1*, but not of *CfPRF1*. These co-regulated genes that define the *CfPKS1* gene cluster are orthologous to genes involved in 1,3-dihydroxynaphthalene (DHN) melanin biosynthesis in other fungi. Heterologous expression of *CfPKS1* in *Aspergillus oryzae* yielded 1,3,6,8-tetrahydroxynaphthalene, a typical precursor of DHN melanin.  $\Delta cfps1$  deletion mutants showed similar altered pigmentation to wild type treated with DHN melanin inhibitors. These mutants remained virulent on tomato, showing this gene cluster is not involved in pathogenicity. Altogether, our results showed that the *CfPKS1* gene cluster is involved in the production of DHN melanin and suggests that elsinochrome production in *E. fawcettii* likely involves another gene cluster.

## Introduction

Secondary metabolites (SMs) are compounds produced by microbes, plants and insects that are often repurposed as medicines and pesticides. Equally important are SMs with harmful effects, such as mycotoxins and pathogenicity factors that poison animals or promote crop diseases. The vast majority of fungal SMs with a clear biological role *in situ* are pathogenicity or

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virulence factors, also known as effector SMs, which are produced by plant pathogens during infection of their respective hosts [1,2]. Non-specific toxins synthesised by hemi-biotrophic and necrotrophic fungi are compounds that necrotise host tissues indiscriminately, whilst host-selective toxins (HSTs) only cause necrosis on plants expressing corresponding susceptibility genes, thereby determining host range [2].

1,8-dihydroxynaphthalene (DHN) melanin is a virulence SM for several plant and human fungal pathogens. DHN melanin is required for the penetration of rice leaves by *Magnaporthe oryzae*, a process mediated by appressoria, dome-shaped cells specialized in piercing the plant cuticle and cell wall [3]. Failure to melanise the fungal cell wall results in immature appressoria that cannot generate the turgor pressure required to penetrate host tissues [4,5]. Tricyclazole, pyroquilon and other commercial compounds that inhibit DHN melanin biosynthesis are highly effective at preventing rice blast [6–8]. The same role in plant penetration was reported in several other plant pathogens, including *Colletotrichum kahawae* and *Diplocarpon rosae*, pathogens of coffee berries and roses, respectively [9,10]. It has been suggested that DHN melanin is also a photodynamic virulence factor used by *Pseudocercospora fijiensis*, the causal agent of black Sigatoka disease, to generate toxic reactive oxygen species during infection of banana [11]. In addition to its role in virulence, DHN melanin provides tolerance to many kinds of abiotic stresses, including radiation and extreme temperatures [9,10]. Accordingly, DHN melanin production is often linked to the development of survival structures. For example, DHN melanin accumulates in the cell wall of conidia and sclerotia of the plant pathogen *Botrytis cinerea*, but it does not play a role in the virulence of this pathogen [12]. In the plant endophytic fungus *Pestalotiopsis fici*, DHN melanin was recently shown to be essential for the development of multicellular conidia [13].

Fungal DHN is produced through a polyketide pathway, which starts with a non-reducing polyketide synthase (nrPKS) [14,15]. The first stable intermediate, 1,3,6,8-tetrahydroxynaphthalene (4THN), can be produced through three distinct biosynthetic routes. In Sordariomycetes such as *Colletotrichum lagenarium* [16,17], the nrPKS carries a bi-functional release domain that produces the hexaketide acetyl THN (ATHN) through Claisen ring closure, and then deacetylates ATHN to release the pentaketide 4THN [18]. The nrPKS in the Eurotiomycete fungus *Exophiala dermatitidis* also releases ATHN [19], but the deacetylation step is instead performed by the discrete hydrolase, YG1 [20]. In other Eurotiomycetes fungi such as *Aspergillus* and *Penicillium* species, the nrPKS is a heptaketide synthase that releases YWA1 [21–23], which is deacylated by the hydrolase AYG1 to produce 4THN [21,24,25]. In certain fungal species like *B. cinerea*, two nrPKSs, likely one synthase with a bi-functional release domain (BcPks12) and one hexaketide or heptaketide synthase (BcPks13), are involved in DHN melanin biosynthesis [12]. The subsequent enzymatic steps to convert 4THN to DHN are common to all fungal species; 4THN is first reduced to scytalone by a 4HNR reductase, then dehydrated to 1,3,8-trihydroxynaphthalene (3THN) by the dehydratase SCD1 [15]. 3THN is reduced to vermelone by the reductase 3HNR, then dehydrated by SCD1 to yield DHN [15]. These reductases, especially 3HNR, are the target of tricyclazole [26]. Finally, DHN is polymerized into melanin by multicopper oxidases [27–30]. These different pathways have been invoked to explain the difference in pigmentation between brown-black fungi, including *C. lagenarium*, *M. oryzae* and *C. heterostrophus*, and blueish-green fungi like *A. fumigatus* that might polymerize YWA1 in addition to DHN [30]. The genes encoding enzymes involved in DHN melanin biosynthesis and polymerization are organized in a gene cluster in *A. fumigatus* [30], and *Penicillium marneffei* [31], but they are partially clustered in *Alternaria alternata* and *Cochliobolus heterostrophus* [32,33] and tend to be dispersed in other fungal genomes [15].

It must be noted that certain fungal species produce another kind of melanin that is synthesized from L-3,4-dihydroxyphenylalanine (DOPA) through the action of tyrosinases and

laccases [34]. Although the DHN melanin genes can be present in fungal genomes, the DOPA melanin pathway is the major route employed by certain fungal species, as exemplified by the pine needle pathogen *Dothistroma septosporum* [35].

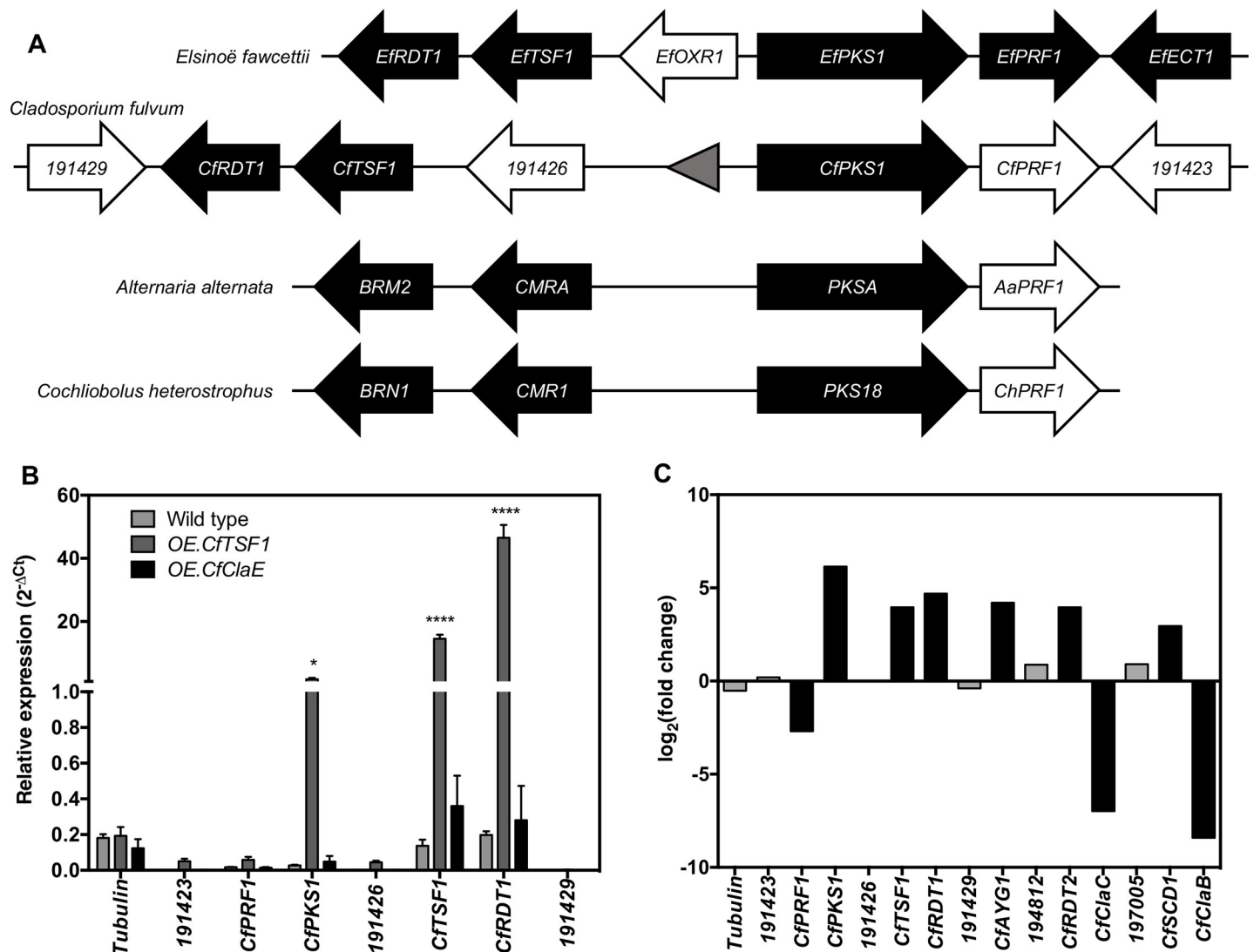
*Cladosporium fulvum* is a non-obligate, biotrophic fungus that causes tomato leaf mold disease. *C. fulvum* shows limited filamentous growth on *in vitro* media in the dark, forming small sporulating colonies. They exhibit a green-brown colour, which was linked to the production of the pigment cladofulvin [36]. *C. fulvum* colonies harbour a grey colour when cladofulvin is not produced, which is likely due to the production of another pigment [36]. *C. fulvum* is known to reproduce asexually only and production of cladofulvin is primarily observed in conidia [37]. Despite a high potential chemical diversity with 23 predicted-functional SM core genes [38,39], the pigment cladofulvin produced by the *claG* gene cluster remains the only detectable SM [36,40]. It was suggested and later shown that the repression of cladofulvin biosynthetic genes is required for biotrophic growth of *C. fulvum* [37,38]. *CfPKS1* is another nrPKS core gene that shows a similar expression profile during infection of tomato leaves, *i.e.* downregulation [38]. *CfPKS1* belongs to a predicted gene cluster containing genes that encode a prefoldin chaperone (*CfPRF1*), a reductase (*CfRDT1*) and a transcription factor (*CfTSF1*) [38]. Previous comparative genomic analyses indicated that the *CfPKS1* gene cluster is homologous to the *Elsinoë fawcettii* gene cluster responsible for elsinochrome production, a light-activated toxin involved in the virulence of this pathogen on citrus hosts [41,42]. However, the phylogeny of *CfPKS1* suggested that it is also closely related to nrPKSs involved in DHN melanin biosynthesis [38]. In another study, *CfPKS1* was strongly up-regulated in the *C. fulvum* deletion mutant  $\Delta cfwor1$  during growth on agar [43]. The hyper-black appearance of  $\Delta cfwor1$  colonies and the absence of detectable SMs suggested that *CfPKS1* might be involved in the production of polymerized DHN melanin in *C. fulvum*. Such ambiguity between elsinochrome and DHN production remains unresolved.

Here, we report the functional characterization of the *CfPKS1* gene cluster by targeted gene deletion (*CfPKS1*), over-expression of the predicted local regulator (*CfTSF1*), and heterologous expression in *Aspergillus oryzae*. We provide chemical evidence of the pigment produced by this pathway and assessed the role of this compound in pathogenicity and biotrophic growth of *C. fulvum*.

## Results

### Definition of the *CfPKS1* gene cluster in *Cladosporium fulvum*

The *CfPKS1* gene cluster (Fig 1A) was initially predicted solely through its homology and synteny with the characterized elsinochrome gene cluster in *E. fawcettii*, as only minimal gene expression within this gene cluster had been observed during the growth of wild-type *C. fulvum* under diverse conditions [38]. The gene cluster includes *CfTSF1*, a gene predicted to encode a pathway-specific transcription factor [38,41]. To up-regulate and clearly define the *CfPKS1* gene cluster, wild-type *C. fulvum* was transformed with a plasmid containing *CfTSF1* fused to the promoter region of the nitrogen-regulated *C. fulvum* *Avr9* gene [44]. The resulting *OE.CfTSF1* transformant (S1 Fig) does not show any *in vitro* difference compared to wild type, but this transformant is not pathogenic on tomato (S2 Fig). Although random insertion of the expression cassette in a pathogenicity gene cannot be excluded, this loss of pathogenicity is likely due to the up-regulation of *CfTSF1* because the *Avr9* promoter induces high-expression when *C. fulvum* enters the plant and colonizes leaf tissues [38,44]. Both transformant and parental strain were grown in PDB and then induced in B5 medium without nitrogen (B5-N) for 48 hours to induce gene expression. Transcriptional profiling by RT-qrtPCR showed that the relative expression of *CfPKS1*, *CfTSF1* and *CfRDT1* was 1.7, 14.6 and 46.5-fold higher,



**Fig 1. Definition of the *CfPKS1* gene cluster in *Cladosporium fulvum*.** (A) Organization of the predicted *CfPKS1* gene cluster based on the reported homologous gene cluster in *Elsinoë fawcettii* [38,41]. The locus of the DHN melanin gene cluster in *Alternaria alternata* and *Cochliobolus heterostrophus* is shown for comparison [32,33]. All loci contain a prefoldin-encoding gene downstream of the polyketide synthase gene. *CMR1* and *CMRA* encode transcription factors that regulate the DHN melanin biosynthetic genes. *BRN1* and *BRM2* are 3-hydroxy naphthalene reductases homologous to *RDT1*. Black arrows indicate co-regulated genes in each species, white arrows indicate non-co-regulated genes and the grey triangle indicate a transposable element. Loci are not drawn to scale. (B) Relative expression of genes at the *CfPKS1* locus determined by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). Strain were pre-cultured in Potato Dextrose Broth (PDB) for five days before the biomass was transferred to B5 without nitrogen (B5-N) medium. After 48 hours, the biomass was recovered and used for RNA isolation and cDNA synthesis. The expression value for each gene within the *CfPKS1* locus was measured in wild-type *C. fulvum* and inducible over-expression transformants *C. fulvum* OE. *CfTSF1* and OE.*CfClaE* [38] grown in B5-N medium. Expression values were normalised to *actin* and the average value was plotted with standard deviation between three biological replicates. A two-way ANOVA with a posthoc Sidak multicomparison test at the significance level of 0.05 was used to calculate statistically significant changes in gene expression between wild type and transformant strains. Asterisks (\*) denote statistically significant changes ( $p < 0.05$  or less). (C) Differential expression of genes at the *CfPKS1* locus and homologues of genes involved in the biosynthesis of DHN melanin in the  $\Delta cfw1$  deletion mutant compared with the wild type as determined by RNA-seq [43]. Black and gray bars show significant and nonsignificant fold changes, respectively, according to Cuffdiff analysis of three biological repeats.

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respectively, in the OE.*CfTSF1* transformant than in wild type (each t-test P-value  $< 0.0001$ ), whilst the predicted border genes are not co-regulated (Fig 1B). In contrast, *CfPRF1* was not co-regulated and is therefore unlikely part of the *CfPKS1* biosynthetic pathway. The up-regulation of the *CfPKS1* gene cluster is specific to the over-expression of *CfTSF1* because the gene



cluster is not significantly activated when the regulator of cladofulvin production (*CfClaE*; 40) is over-expressed (Fig 1B).

In a previous study, the *CfPKS1* core gene was found to be highly up regulated in  $\Delta cfwor1$  deletion mutants [43]. The published RNA-seq data of this mutant confirms that all genes from the predicted gene cluster are co-regulated, but *CfPRF1* and the predicted border genes are not (Fig 1C). Consistent with previous phylogenetic analyses of 4THN synthases [35,38,45], *CfPKS1*, *CfRDT1* and *CfTSF1* are all orthologous to genes involved in DHN melanin biosynthesis (Figs 2A–2C). In other fungi, this pathway involves three other genes, *AYG1*, *4HNR* and *SCD1* [12,34]. Orthologues of these genes were identified on different scaffolds in the genome of *C. fulvum* (Table 1; Figs 2C–2E) and all are significantly up-regulated in the  $\Delta cfwor1$  deletion mutants (Fig 1C). In contrast, paralogues of these genes are not differentially expressed or are significantly down-regulated in the  $\Delta cfwor1$  deletion mutants (Fig 1C).

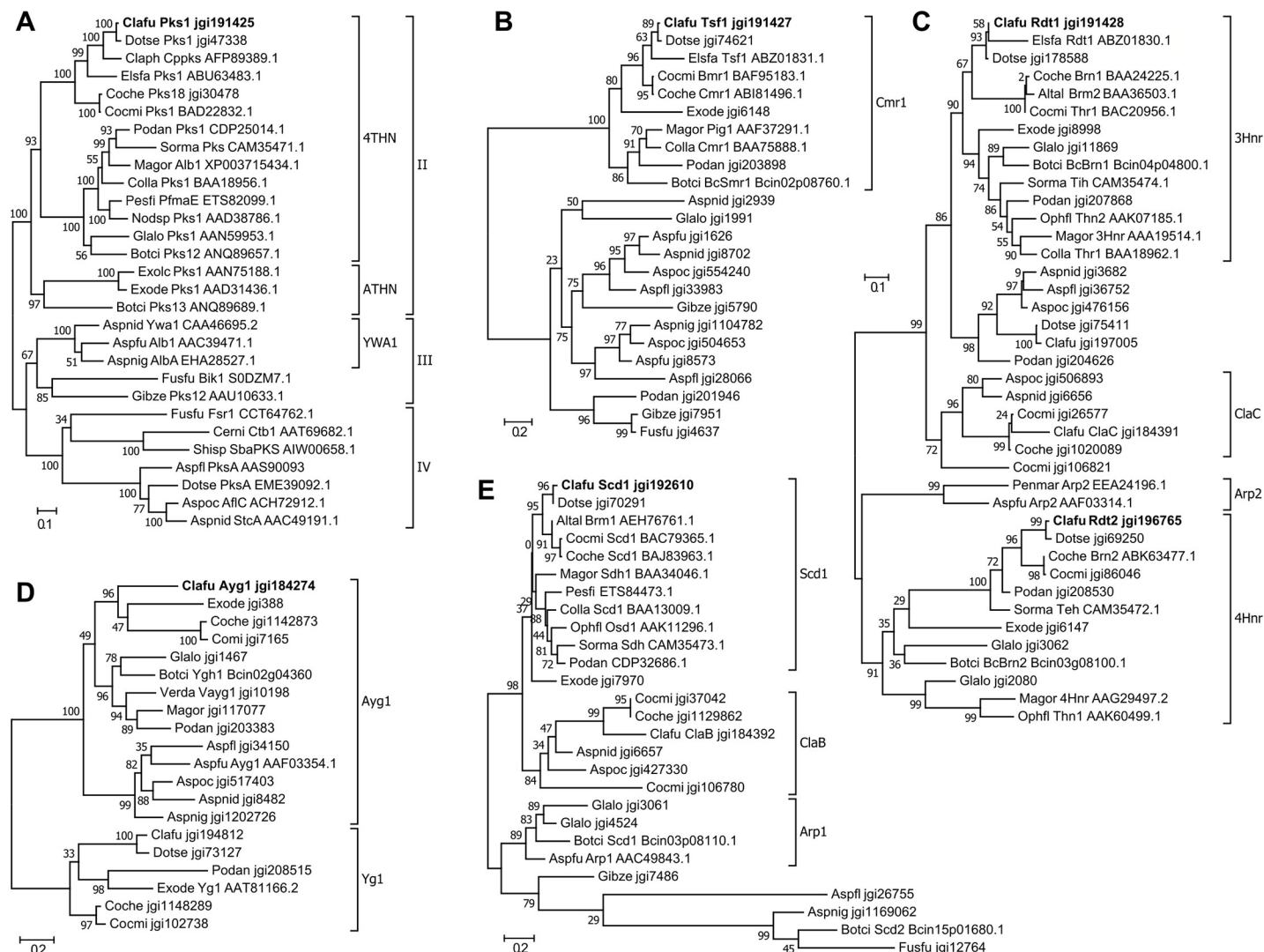
### CfPks1 is a polyketide synthase that releases 4THN

*CfPKS1* is orthologous to other characterized 4THN synthases from fungi of different orders (Fig 2A). In *C. lagenarium*, the 4THN synthase ClPks1 carries a bi-functional TE domain that releases 4THN [18]. CfPks1 was expressed heterologously in *A. oryzae* M-2-3 in order to determine whether or not it catalyses the same reactions as ClPks1. Ethyl acetate extracts of transformants contained three major products, 1–3, bearing UV signatures diagnostic of aromatic polyketides (Fig 3). Product 1 (RT = 4.9 min; UV max = 210, 261, 307 nm;  $m/z$  ( $ES^+$ ) 205 [M-H] $^+$ ) was identified as flaviolin by comparing its UV and mass spectra to published data (S3 and S4 Figs) and was confirmed by High-Resolution Mass Spectrometry (HRMS; exact mass 207.0283; S5A Fig). Flaviolin is a spontaneously oxidised degradation product of 4THN. Product 2 (RT = 5.4 min; UV max = 244, 327 nm;  $m/z$  ( $ES^+$ ) 233 [M-H] $^+$ ) harbours the same chemical formula as benzopyran according to HRMS (exact mass 235.0600; S5B Fig), which is a compound known as a shunt metabolite of the 4THN hexaketide pathway [18]. Product 3 (RT = 6 min; UV max = 244, 325 nm;  $m/z$  ( $ES^+$ ) 191 [M-H] $^+$ ) was identified as 4THN by HRMS (exact mass 193.0498; S5C Fig). These results clearly show that CfPks1 produces the same intermediate as ClPks1 and thus exhibits the same enzymatic activity.

LC-MS analyses of organic extracts obtained from *OE.CfTSF1* transformant after 48h growth on induction medium did not detect any precursor of DHN melanin. This observation could be due to a delay between the transcriptional induction and production of the compounds in significant amount as there was no pigmentation difference with wild type. Alternatively, it could suggest that DHN is efficiently polymerized, which is not easily extractable from cell walls with regular chemical methods.

### *CfPKS1* is needed for proper pigmentation of *C. fulvum*, but it is not required for pathogenicity on tomato

To confirm the heterologous expression results and obtain further insights in the biological role of the *CfPKS1* gene cluster, the *CfPKS1* gene was replaced by a deletion cassette containing the hygromycin resistance marker gene *via* homologous recombination (S1 Fig). Two confirmed independent deletion mutants and an ectopic transformant were selected for further analysis. Both  $\Delta cfpk1$  deletion mutants were yellow-orange compared to the grey-green ectopic transformant and wild type (Fig 4A). The wild-type strain shows similar coloration to the  $\Delta cfpk1$  deletion mutants when it is grown in the presence of pyroquilon, an inhibitor of the DHN melanin pathway (Fig 4A; [7]). In contrast, it was not coloured differently when grown in the presence of hydroquinone, an inhibitor of DOPA melanin (Fig 4A; [7]). These results contrast with the situation in *D. septosporum*, a close relative species of *C. fulvum* that



**Fig 2. Phylogenetic analysis of proteins involved in DHN melanin biosynthesis.** Maximum likelihood phylogenetic trees were built to resolve the evolutionary relationship of (A) Cfpks1 to characterized non-reducing polyketide synthases; (B) Cfts1 to characterized regulators of DHN melanin biosynthesis; (C) Cfrdt1 and Cfrdt2 to reductases involved in the reduction of 1,3,8-trihydroxy- and 1,3,6,8-tetrahydroxy-naphthalene, respectively; (D) Cfaig1 to characterized hydrolases involved in polyketide deacetylation; and (E) Cfscl1 to characterized scytalone dehydratases. *Elsinoe fawcettii* proteins assigned to the elsinochrome gene cluster are included [41]. Non-characterized homologues are also included for species in which the polyketide synthase involved in DHN synthesis is characterized. The name of characterized proteins and accession numbers (GenBank, SwissProt or Joint Genome Institute (JGI) protein ids) are indicated next to the species acronym. Cladu: *Cladosporium fulvum*; Dotse: *Dothistroma septosporum*; Claph: *Cladosporium phlei*; Elsfa: *Elsinoe fawcettii*; Coche: *Cochliobolus heterostrophus*; Cocmi: *Cochliobolus miyabeanus*; Podan: *Podospira anserina*; Sorma: *Sordaria macrospora*; Magor: *Magnaporthe oryzae*; Colla: *Colletotrichum lagenarium*; Pesfi: *Pestalotiopsis fici*; Nodsp: *Nodulisporium sp.*; Glalo: *Glareae lozoyensis*; Botci: *Botrytis cinerea*; Exolc: *Exophiala lecano-cani*; Exode: *Exophiala dermatitidis*; Aspnid: *Aspergillus nidulans*; Aspfu: *Aspergillus fumigatus*; Aspnig: *Aspergillus niger*; Fusfu: *Fusarium fujikoro*; Gibze: *Gibberella zeae*; Cerni: *Cercospora nicotianae*; Shisp: *Shiraia sp.*; Aspfli: *Aspergillus flavus*; Aspoc: *Aspergillus ochraceus*; Altal: *Alternaria alternata*; Ophfl: *Ophiostoma floccosum*; Penmar: *Penicillium marneffe*; Verda: *Verticillium dahliae*.

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produces DOPA melanin despite the presence of *DsPKS1*, the orthologue of *CfPKS1* and other 4THN synthase genes [35]. Together, these results show that the polymerization of DHN melanin contributes to the pigmentation of *C. fulvum*. The  $\Delta cfpks1$  mutants do not manifest any other obvious developmental or physiological defects.

Because DHN melanin is a pathogenicity factor in several plant pathogens, we addressed the possibility that this role was also true in *C. fulvum*. Tomato plants were inoculated with wild-type *C. fulvum*, an ectopic transformant and two independent  $\Delta cfpks1$  deletion mutants.

Table 1. List of *Cladosporium fulvum* genes that are orthologous to melanogenic genes in other fungi.

Gene name	Protein number <sup>a</sup>	Scaffold	Position	Function	Conserved domains <sup>b</sup>
<i>CfPKS1</i>	191425	scf7180000130411	192,236–198,793	Non-reducing polyketide synthase	PF16073 SAT PF00109/PF02801 KS PF00698 AT PF14765 (dehydratase) PT PF00550 ACP PF00550 ACP PF00975 TE
<i>CfTSF1</i>	191427	scf7180000130411	211,934–215,018	Fungal specific transcription factor	pfam00172 Fungal Zn(2)-Cys(6) binuclear cluster domain PF04082 Fungal specific transcription factor domain
<i>CfRDT1</i>	191428	scf7180000130411	217,755–218,741	3-hydroxy naphthalene reductase	PF13561 Enoyl-(Acyl carrier protein) reductase
<i>CfAYG1</i>	184274	scf7180000126929	44,831–46,097	hydrolase	PF06500 Alpha/beta hydrolase of unknown function
<i>CfRDT2</i>	196765	scf7180000130934	32,438–33,312	4-hydroxy naphthalene reductase	PF13561 Enoyl-(Acyl carrier protein) reductase
<i>CfSCD1</i>	192610	scf7180000130653	83,529–84,393	Scytalone dehydratase	PF02982 Scytalone dehydratase

<sup>a</sup> Joint Genome Institute accession number

<sup>b</sup> Determined using the PFAM database; the typical domains of polyketide synthases are indicated for *CfPks1*. SAT: Starter unit:ACP transacylase; KS: Ketoacyl Synthase; AT: Acyl Transferase; PT: Product Template; ACP: Acyl Carrier Protein; TE: ThioEsterase. Note that the PT domain is not present in the PFAM database and instead is related to a dehydratase domain.

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Whilst the growth of all strains was similar at 4 and 8 dpi, the *Δcfpks1* deletion mutants had significantly outgrown the control strains by 12 dpi (Fig 4B). By 16 dpi, this difference in growth became strikingly clear, as the white-orange deletion mutants had colonized a greater leaf surface area than the control strains (Fig 4C).

## Discussion

### The *CfPKS1* gene cluster is involved in DHN melanin biosynthesis

A previous phylogenetic analysis suggested that *CfPKS1* is an orthologue of *EfPKS1*, which encodes the nrPKS required for elsinochrome production [35,38,42]. The predicted elsinochrome gene cluster is also present in *C. fulvum*, except for the putative transporter *ECT1*

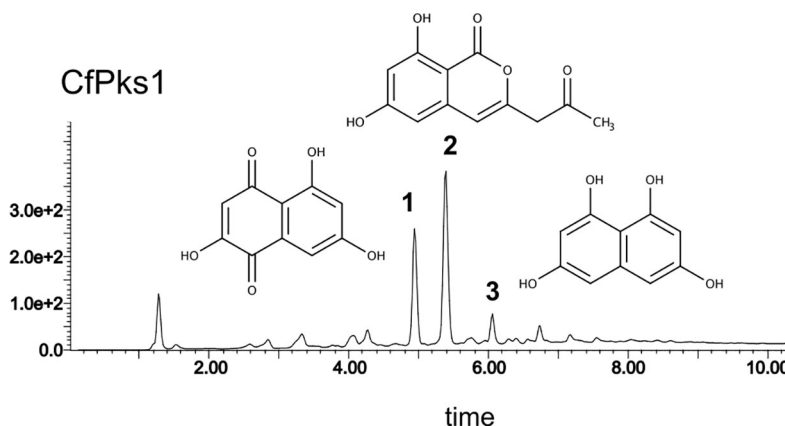
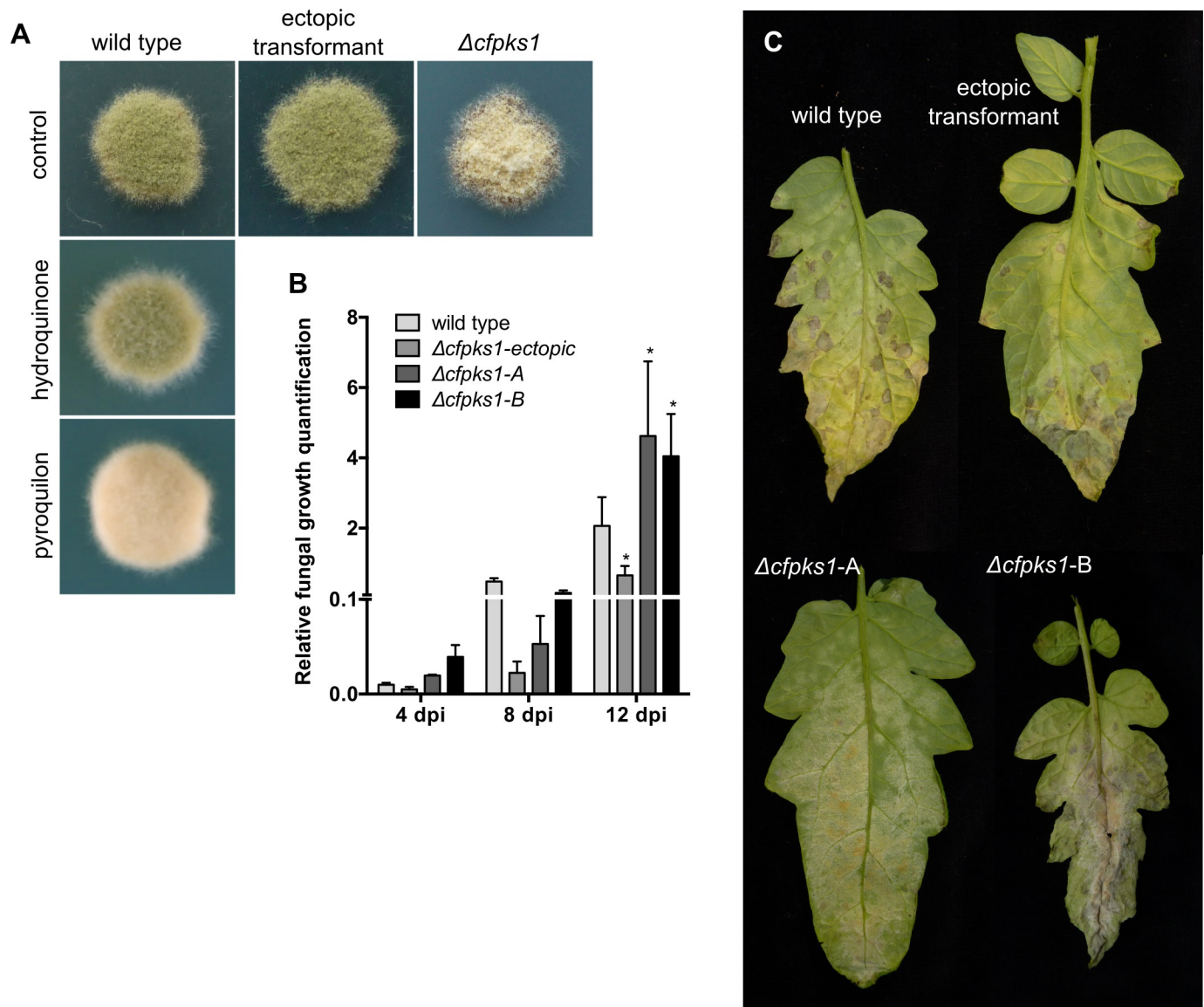


Fig 3. Heterologous expression of *CfPks1* in *Aspergillus oryzae*. Representative diode array chromatogram of ethyl acetate extracts from transformants expressing *CfPks1*. These transformants produced three major compounds that were determined as flaviolin 1, benzopyran 2 and 1,3,6,8-tetrahydroxy-naphthalene (4THN) 3.

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**Fig 4. Characterization of *Cladosporium fulvum*  $\Delta cfps1$  deletion mutants.** (A) *In vitro* growth of wild-type *C. fulvum*, ectopic transformant and  $\Delta cfps1$  deletion mutants on Potato Dextrose Agar (PDA) medium; and wild-type *C. fulvum* on PDA supplemented with 30 mg.L<sup>-1</sup> DHN biosynthesis inhibitor (pyroquilon) or DOPA melanin biosynthesis inhibitor (hydroquinone) [7]. (B) Quantification of fungal growth during tomato infection by wild type, an ectopic transformant and  $\Delta cfps1$  deletion mutants. (C) Tomato leaves infected by wild type, an ectopic transformant and  $\Delta cfps1$  deletion mutants at 16 days post-inoculation (dpi).

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[38,41]. However, the same phylogenetic analysis showed that *CfPKS1* and *EfPK1* are orthologous to 4THN synthases involved in the biosynthesis of DHN melanin. Similar observations were reported in other phylogenetic analyses [35,45]. Given that PKSs from a monophyletic clade tend to produce the same products, the different chemical structures of DHN melanin and elsinochrome contradict the phylogeny of their respective nrPKSs. The orthologous 4THN synthase in *C. lagenarium* has been characterized in detail; ClPKs1 synthesizes the hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (ATHN) that is cyclized and deacylated by its bi-functional TE domain to give 4THN [18]. In this report, we showed that *C. fulvum* CfPKs1 is also a synthase that releases 4THN because it produced both the hexaketide benzopyran and

the pentaketide 4THN (Fig 3). The precursor ATHN was not detected in our experiments, which might be due to the presence of an expressed homologue of the hydrolase Ayg1 in *A. oryzae* that would efficiently deacetylate ATHN to yield 4THN [18, 20].

In characterized nrPKSs involved in DHN melanin biosynthesis, aromatisation of the nascent ACP-bound polyketide to give a monocyclic intermediate is catalysed by the product template (PT) domain [14,16,18,46]. Closure of the second ring and release of the polyketide requires the Claisen/Dieckmann cyclase class of TE domains (TE/CLC) [14,18,46]. The absence of a functional TE domain results in the release of pyrone shunt products *via* spontaneous O-C cyclization [18]. The co-detection of 4THN and benzopyran in *A. oryzae*::*CfPKS1* suggests that the TE domain of *CfPKS1* was not fully functional, which might be due to the absence of tailoring enzymes.

Ayg1 and its orthologues were shown to be involved in the deacetylation of a hexaketide or heptaketide precursor in the DHN melanin pathway described in blue/green fungi [24]. However, it was recently shown in the plant pathogen *V. dahliae* that *AYG1* might also be important for DHN biosynthesis in brown/black fungi [47]. Our finding that *CfAYG1* is present in *C. fulvum* genome and co-regulated with *CfPKS1* suggests that this gene also plays a role in the DHN biosynthetic pathway in *C. fulvum*, which requires further investigation. Although another paralogue is present in *C. fulvum*, it is not co-regulated with *CfPKS1* and thus is unlikely to be involved in 4THN biosynthesis (Figs 1 and 2). It is noteworthy that Yg1 in *E. dermatitidis* is actually a paralogue of characterized Ayg1 in other fungal species (Fig 2). The true orthologue of Ayg1 in *E. dermatitidis* must also be further investigated to ascertain its involvement in 4THN production.

Our phylogenetic analysis showed that co-regulated genes at the *CfPKS1* locus in *C. fulvum* and genes from the predicted elsinochrome gene cluster (*PKS1*, *RDT1* and *TSF1*) in *E. fawcettii* are orthologous to genes involved in DHN biosynthesis (Fig 2). A gene cluster for elsinochrome biosynthesis was recently characterized in *Stagonospora nodorum* [45]. This gene cluster was shown to be related to gene clusters involved in the biosynthesis of cercosporin, a compound of the perylenequinone family that is structurally related to elsinochrome [48–50]. Together with our characterization of *CfPKS1* as a 4THN synthase, these observations suggest that the elsinochrome gene cluster in *E. fawcettii* has not been accurately assigned. Instead, a cercosporin-like gene cluster is certainly involved in the production of elsinochrome as reported in *S. nodorum*. The fungus *Cladosporium phlei* produces phleichrome, a perylenequinone that is structurally related to elsinochrome and cercosporin [48]. The *C. phlei* nrPKS gene *CpPks1*, orthologous to *CfPKS1* and *EfPKS1*, was assigned to phleichrome production [51]. Similarly, we suggest that *CpPks1* is involved in DHN melanin production and another cercosporin-like nrPKS is responsible for phleichrome production. Such false assignments suggest that crosstalk and interdependencies between gene clusters might be more important than previously thought [52].

### DHN melanin is not a virulence factor in *C. fulvum*

The biosynthesis of fungal DHN melanin has been extensively studied because of its diverse roles in fungal biology [15]. Melanin contributes to virulence in animal and plant pathogens, with the latter linked to the formation of host-invading appressoria [15]. In species that do not produce appressoria, melanin confers resilience to chemical and abiotic stresses [12]. *C. fulvum* does not differentiate appressoria, which likely explains why DHN melanin is not involved in the pathogenicity of this fungus. The pigmentation of *C. fulvum* relies on two compounds, the greyish DHN melanin and yellow-orange-purple (depending on pH) cladofulvin [36,40]. Cladofulvin is also not produced during plant infection, but instead protects the

fungus from environmental stresses such as UV light and cold temperatures [37]. Although untested, DHN melanin likely plays a similar role in the survival of *C. fulvum ex planta*.

Although further investigation is required to fully exclude that the expression cassette has inserted within a pathogenicity gene, the observed loss of pathogenicity of the *OE.CfTSF1* transformant suggests that adequate regulation and downregulation of DHN melanin production during leaf colonization is required for full pathogenicity in *C. fulvum*. In *A. fumigatus* and *A. nidulans*, melanin biosynthesis is initiated in endosomes that carry the enzymes that produce DHN, which is then polymerized within the cell wall by multicopper oxidase and laccase enzymes [53]. Melanin is polymerised in the cell wall, forming several layers of globular particles that grow thicker over time, strengthening the cell wall [54]. It is accepted that hyphal tip elongation requires enzymes to weaken the cell wall in order to incorporate new components, which are then cross-linked to rigidify the cell wall [55]. Thus, the abnormal accumulation of melanin in fungal cell walls is likely to modify its physical properties and increases its rigidity. We observed that the runner hyphae of *C. fulvum OE.TSF1* transformants branched infrequently and the few successful penetration events resulted in colonizing hyphae that rapidly stopped growing (S2 Fig). This phenotype can be explained by the elevated production of DHN melanin and its abnormal accumulation in cell walls, which might reduce sensitivity to chemotactic gradients and then arrest growth of colonizing hyphae by preventing essential fungal tip remodelling [55]. An *in planta* analysis of fungal cell walls would address this hypothesis. Alternatively, DHN melanin accumulating in the cell wall could be recognized by plant cells, leading to the activation of plant defences and resistance. Similar *in planta* activation might be detrimental to the virulence of other plant pathogens irrespective of whether or not DHN melanin is a pathogenicity factor.

## Conclusion

Using complementary approaches (gene expression, phylogeny, heterologous expression), our study confidently assigned the *CfPKS1* gene cluster to DHN melanin in *C. fulvum*. It suggests that orthologous gene clusters in other species have been wrongly assigned to toxin production, including elsinochrome in *E. fawcettii* and phleiochrome in *C. phlei*. Further investigations in these fungal species are needed to address this ambiguity and are likely to provide important insights on pathway crosstalk that might lead to incorrect gene cluster assignment.

## Experimental procedures

Most of the methods were performed as described in Griffiths *et al.* (2016) and Griffiths *et al.* (2018) [37,40].

## Fungal strains employed in this study

*C. fulvum* 0WU [39] was the parental strain used to perform transformation and gene deletion experiments. *C. fulvum* was grown on potato dextrose agar (PDA) plates at 20°C in the dark. For inhibitor experiments, PDA medium was supplemented with 30 mg.L<sup>-1</sup> pyroquilon or hydroquinone (Sigma-Aldrich, Zwijndrecht, The Netherlands). *A. oryzae* M-2-3 strain was used to perform heterologous expression [56].

## Phylogeny

The protein sequence of CfPks1 and of selected characterized nrPKSs from groups II, III and IV [57] were aligned using Muscle [58] and poorly aligned regions of the alignment were removed using Gblocks, with half allowed gap positions for Ayg1, Rdt and Scd1 alignments, and allowing

all gapped positions for the Tsf1 alignment [59]. Maximum-likelihood phylogeny was calculated using PhyML 3.1 [60] with the LG+G+I substitution model as determined by Modelgenerator v851 [61] and SH approximate likelihood ratio test to evaluate branch support.

Non-characterized homologues were retrieved from the Joint Genome Institute MycoCosm portal (genome.jgi.doe.gov; [62]) using BlastP (with default parameters) [63]. All Homologues of 4Hnr (AAG29497.2) and Sdh1 (BAA34046.1) from *M. oryzae* and of Ayg1 (AAF03354.1) from *A. fumigatus* were sought in the predicted proteome of *C. fulvum*. Homologues of CfRdt1, CfTsf1, CfRdt2, CfScd1 and CfAyg1 were sought in selected genomes. For each protein, retrieved homologues and characterized proteins were aligned using Muscle; sequences with large deletions or insertions were manually removed. Neighbour-Joining phylogenetic trees were built with the JTT substitution model using MEGA 7 [64] in order to ascertain orthology. Identified orthologues were then aligned again and analysed following the same process as for CfPks1 described above, but using the LG+G substitution model for Ayg1 and Scd1 trees.

### Generation of *OE.CfTsf1* and *OE.CfTsf1::GFP* transformants

The putative local regulator from the *CfPKS1* gene cluster, *CfTsf1*, was amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (Life Technologies, Carlsbad, CA) from *C. fulvum* genomic DNA using the primer pair *PacI\_CfTsf1\_Forward* and *NotI\_CfTsf1\_Reverse* (S1 Table). Plasmid *pFBTS3* contains the promoter of the nitrogen-regulated *Avr9* gene [36,44]. The *CfTsf1* amplicon and *pFBTS3* were cut using *PacI* and *NotI* restriction enzymes (Fermentas Fast Digest, Waltham, MA), cleaned with Zymogen DNA Clean & Concentrator (Baseclear, Leiden, The Netherlands), and ligated using T4 DNA polymerase (Promega, Madison, WI) to yield *pFBTS3-CfTsf1*. *Escherichia coli* DH5 $\alpha$  cells were transformed with the ligation mix using a standard heat-shock protocol and transformants were selected in lysogeny broth (LB)-kanamycin agar (50  $\mu\text{g}.\text{ml}^{-1}$ ). Plasmids were extracted from transformants and screened by restriction digest analysis using *PacI* and *NotI* in a double digestion. A plasmid bearing the correct restriction pattern was sent to Macrogen (Amsterdam, The Netherlands) for sequencing of the insert. *Agrobacterium tumefaciens* AGL1 was transformed with *pFBTS3-CfTsf1* by electroporation, and plated on LB-kanamycin agar (50  $\mu\text{g}.\text{ml}^{-1}$ ). One positive transformant was picked, verified and named *AT-pFBTS3-CfTsf1*. This plasmid was introduced to *C. fulvum* using *A. tumefaciens*-mediated transformation as previously described [65]. Transformants were selected on PDA medium supplemented with hygromycin (100  $\mu\text{g}.\text{ml}^{-1}$ ). Several transformants and wild-type *C. fulvum* were grown for 5 days in potato-dextrose broth (PDB; Oxoid, Altrincham, UK) and then transferred to Gamborg B5 medium without nitrogen (B5-N) in order to induce the *Avr9* promoter [37,44]. Total RNA was extracted and cDNA synthesis was performed as previously described [65]. The induction of the *CfPKS1* biosynthetic gene cluster was confirmed by RT-qrtPCR using primers listed in S1 Table. One transformant showing the expected strong induction of genes at the *CfPKS1* locus was selected and named *OE.CfTsf1*.

Using the same methods, *A. tumefaciens* AGL1 was transformed with plasmid *pRM254*, which contains *GFP* and geneticin-resistance genes [66] to yield *AT-pRM254* strain. The plasmid was introduced into the *OE.CfTsf1* transformant as described above. Transformants were selected on PDA medium supplemented with geneticin (100  $\mu\text{g}.\text{ml}^{-1}$ ). Transformants were picked and screened for *GFP* fluorescence. One transformant was selected and named *OE.CfTsf1::GFP*.

### Generation of $\Delta\text{cfpks1}$ deletion mutants

The plasmid for targeted gene replacement of *CfPKS1* was generated following the same procedure as described in Griffiths *et al.* (2016) [40]. The upstream (US) and downstream (DS)



regions flanking of *CfPKS1* were amplified using primers 1 + 2 and 3 + 4, respectively (S1 Table), and cloned into *pDONR-P4-P1R* and *pDONR-P2-P3* vectors. The final gene replacement plasmid was assembled in a LR reaction (Invitrogen) that combined the *pDONR-P4-P1R::US\_CfPKS1*, *pDONR-P2-P3::DS\_CfPKS1*, *p221\_GFP\_HYG* (*pDONR* containing a cassette with *GFP* and *HYG* resistance marker genes) and the destination vector *pDEST R4-R3* [40,66]. One correct sequenced plasmid was chosen and named *pDest43-Δcfpks1*. This plasmid was introduced into *C. fulvum* 0WU using the *A. tumefaciens* transformation method as described in Okmen *et al.* (2013) [65]. Transformants were selected on PDA plates containing hygromycin (100 μg.mL<sup>-1</sup>). Genomic DNA of each strain was isolated using a Zymo Research Genomic DNA Clean & Concentrator<sup>TM</sup> (Baseclear), according to the manufacturer's recommendations. PCR and quantitative real-time PCR were performed to screen for double cross-overs and measure the number of inserted deletion cassettes, respectively (S1 Fig and S1 Table).

### Plant inoculation and determination of fungal growth

Inoculation of tomato with *C. fulvum* wild-type, deletion mutant and transformant strains was carried out according to a previously described method [67]. To determine fungal growth, the fourth composite leaf of infected tomato plants was harvested at 4, 8, and 12 days post-inoculation (dpi) and flash frozen in liquid nitrogen. Samples were ground to a fine powder in liquid nitrogen, and total RNA was extracted from 100 mg of material using a Zymogen Direct-zol RNA MiniPrep kit (Baseclear) according to the manufacturer's recommended protocol. cDNA synthesis was performed using 100–2,000 ng of total RNA and M-MLV reverse transcriptase (Promega), following the manufacturer's protocol. To assess *C. fulvum* growth during infection, the *actin* gene of this fungus was targeted by qrtPCR using the *Cf-actin\_RT-qrtPCR\_F/Cf-actin\_RT-qrtPCR\_R* primer pair method [67]. For sample calibration, the *Solanum lycopersicum actin* gene was targeted using the *Sl-actin\_qrtPCR\_F/Sl-actin\_qrtPCR\_R* primer pair method [67]. The same cDNA samples were used to measure the expression of genes at the *CfPKS1* locus by qrtPCR using previously reported methods and primers [38]. Additional oligonucleotides (S1 Table) were designed and their efficiency determined as described in [38]. Results were analyzed according to the  $2^{-\Delta C_t}$  method [68] and are the average of three biological replicates.

### Microscopic examination of GFP-expressing strains

Imaging of infected tomato leaves was performed using a spinning disc confocal microscope (Nikon Ti microscope body (Shinagawa, Tokyo, Japan), Yokogawa CSUX1 scanner (Mushino, Tokyo, Japan), Photometrics Evolve camera (Tucson, AZ), Metamorph software (Molecular Devices, Sunnyvale, CA), 491 nm laser line; 60x oil 1.40NA objective). Z-stacks were acquired with an internal spacing of 0.5 μm. All images were processed using Fiji software [69].

### Construction of vectors for heterologous expression and generation of *A. oryzae* M-2-3 transformants

The cloning of *CfPKS1* in heterologous expression vectors was performed as described in Griffiths *et al.* (2016) [40]. Briefly, *CfPKS1* was amplified from *C. fulvum* OE.*CfTsf1* transformant cDNA (grown on B5-N medium) by PCR using primers 5 + 6 (S1 Table) and cloned into *NotI*-linearized *pEYA2* using recombination in *S. cerevisiae* BMA 64 to generate plasmid *pEYA2-CfPKS1* [40,53]. *CfPKS1* was transferred into the expression vector *pTAex3GS* using LR clonase (Invitrogen), and the resulting *pTAex3GS-CfPKS1* plasmid was introduced in *A.*



*oryzae* M-2-3 using PEG-mediated transformation as described in Griffiths *et al.* (2016) [40]. The starch-inducible taka-amylase promoter (*PamyB*) controls the expression of *CfPKS1*. The final vector contains the arginine biosynthesis gene (*argB*) for selection of fungal transformants.

## Secondary metabolite extraction and analysis by LC-MS

Selected *A. oryzae* transformants containing *CfPKS1* were grown on dextrose-peptone-yeast extract (DPY) agar plates at 30°C until the whole plates were covered. The cultures were freeze-dried and then homogenised with a pestle and mortar. The homogenate was resuspended in water, acidified to pH4 with HCl, and then twice extracted with ethyl acetate. The organic phase was recovered and dried under a nitrogen flow. Samples were resuspended in acetonitrile (CH<sub>3</sub>CN), centrifuged at 20,000 x g for 5 min in a microcentrifuge tube and then transferred to a 1 mL clear glass shell vial (WAT025054c).

HPLC analysis with a Waters Symmetry reverse phase 5μm, C18, 100 Å column (WAT046980) was carried out on a Waters 600S system. The sample was eluted with a variable gradient of solvents (A) H<sub>2</sub>O and (B) CH<sub>3</sub>CN (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL.min<sup>-1</sup>. The following gradient was used: 0 min, A (95%); 10 min, A (10%); 12 min, A (10%), 15 min, A (0%), 16 min, A (95%), 20 min, A (95%). UV spectra were obtained using a 996-photodiode array (PDA) detector and analysed with the Waters Empower software.

LC-MS data were obtained using a Waters LC-MS system composed of a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex Kinetex column (2.6 μm, C18, 100 Å, 4.6 × 100 mm) equipped with a Phenomenex Security Guard precolumn (Luna C5 300 Å) eluted at 1 mL.min<sup>-1</sup>. Detection was by Waters 2998 Diode Array detector between 200 and 400 nm and Waters SQD-2 mass detector operating simultaneously in ES+ and ES- modes between 100 *m/z* and 650 *m/z*. Solvents were: A, HPLC grade H<sub>2</sub>O containing 0.05% formic acid; B, HPLC grade MeOH containing 0.045% formic acid; and C, HPLC grade CH<sub>3</sub>CN containing 0.045% formic acid. Gradients were as follows: Kinetex/ CH<sub>3</sub>CN: 0 min, 10% C; 10 min, 90% C; 12 min, 90% C; 13 min, 10% C; 15 min, 10% C. Samples were generally diluted to 1 mg.mL<sup>-1</sup> and 10 μL injected (10 μg). Data capture and analysis, including peak integration, was performed using MassLynx 4.1 software (Waters).

## Semi-preparative LC-MS, compound purification and structure determination

Purification of compounds was achieved using a Waters mass-directed autopurification system comprising a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex Kinetex Axia column (5 μm, C18, 100 Å, 21.2 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C5 300 Å) eluted at 20 mL.min<sup>-1</sup> at ambient temperature. Solvent A, HPLC grade H<sub>2</sub>O + 0.05% formic acid; Solvent B, HPLC grade CH<sub>3</sub>CN + 0.045% formic acid. The post-column flow was split (100:1) and the minority flow was made up with HPLC grade MeOH + 0.045% formic acid to 1 mL.min<sup>-1</sup> for simultaneous analysis by diode array (Waters 2998) and ESI mass spectrometry in positive and negative modes (Waters SQD- 2). Detected peaks were collected into glass test tubes. Combined tubes were evaporated under a flow of dry N<sub>2</sub> gas and weighed. HRMS data were measured using Waters Q-ToF Premier operating in ES<sup>+</sup> mode.

## Supporting information

**S1 Fig. Molecular characterization of *Δcfpks1* deletion mutants and *OE.CfTSP1* transformant.**

(PNG)

**S2 Fig. Pathogenicity assay of the *Cladosporium fulvum* OE.CfTSF1 transformant and microscopic observation of the OE.CfTSF1::GFP over-expression transformant on tomato leaves.**

(PNG)

**S3 Fig. Diode array chromatograms (left) and Total Ion Chromatograms (TICs; right) of ethyl acetate extracts from *Aspergillus oryzae* transformants expressing CfPks1.**

(PNG)

**S4 Fig. UV and MS spectra of major products produced by *Aspergillus oryzae* transformants expressing CfPks1.**

(PNG)

**S5 Fig. High Resolution Mass Spectrometry (HRMS) data measured for products produced by *Aspergillus oryzae* transformants expressing CfPks1.**

(PNG)

**S1 Table. Oligonucleotides used in this study.**

(DOCX)

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